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INHIBITOR OF CATECHOLAMINE-INDUCED FREE FATTY ACID MOBILIZATION FROM THE CALF MIDBRAIN

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Abstract—A substance which inhibits free fatty acid (FFA) mobilization has been isolated from the calf midbrain. The inhibitor suppressed norepinephrine-induced FFA mobilization in adipose tissue slices, and the isolated fat cell preparation; theophylline-stimulated lipolysis in adipose tissue also was inhibited. Simultaneous infusion of norepinephrine and inhibitor in the dog suppressed FFA mobilization and the rise in blood pressure associated with norepinephrine. The inhibitor action on FFA mobilization and on blood pressure was reversible. A parallelism existed between the time of exposure to the inhibitor and the extent of inhibition. Addition of inhibitor at varying time intervals during norepinephrine induced FFA release revealed that inhibition could occur at any time during the mobilization process. A procedure is presented for the purification of the midbrain inhibitor. Some properties of the purified preparation are discussed.

INHIBITION and stimulation of lipid mobilization occur normally in the intact organism, and are influenced by hormonal, nutritional and neural mechanisms.

Many agents inhibit catecholamine-induced lipolysis; Carlson[1] reported the inhibition of NE-induced lipolysis* by nicotinic acid, and Lipson and Naimi[2] demonstrated the inhibition of NE-stimulated FFA mobilization in the dog by nicotinic acid and propanolol. Dole[3] found that several nucleotides and nucleosides inhibited lipid mobilization. The prostaglandins are potent inhibitors of lipid mobilization. Prostaglandin E_1 was shown by Steinberg *et al.*[4] to inhibit NE-induced lipolysis *in vitro*, and by Bergstrom *et al.*[5] to inhibit lipid mobilization *in vivo*. A number of ganglionic-blocking agents also inhibit lipid mobilization *in vivo*, but the effect varies with the drug and the species tested.

An earlier publication from this laboratory[6] indicated that an extract of calf-midbrain contained an inhibitor of FFA mobilization. This paper presents some of the properties of the inhibitor and its action in a system *in vitro* and in the intact animal.

MATERIALS AND METHODS

Preparation of midbrain extract

Fresh calf midbrains (free of the pituitary gland and the pituitary stalk) were excized at the slaughterhouse and immediately placed in cold acetone–0·01 M HCl (80:20, v/v). A 13 per cent homogenate of the tissue was made with the acetone–HCl mixture, and adjusted to pH 4·0. After standing at 4° for 16–18 hr to precipitate

* Abbreviations: NE, norepinephrine; FFA, free fatty acids.

protein, the homogenate was centrifuged at 10,000 rev/min for 30 min in an International Refrigerated Centrifuge, model B-20. The clear supernatant was collected and concentrated to dryness on a rotary evaporator at 30°. The concentrated material was shaken with 2 vol. of methanol until a homogeneous suspension was attained; glass beads were used to aid mixing. The mixture was filtered to remove salts and additional protein. Methanol was removed from the filtrate under vacuum. The dried extract was taken up in water; it had a brownish-yellow color, with a pH of 5·6–5·8. In a typical experiment, a batch of midbrains, weighing 17 lb. after processing, was made up in water to a final volume of 56 ml.

Column chromatography

Sephadex. Sephadex G-25 (Pharmacia Fine Chemicals, Inc.) was allowed to swell with water at 4° for 24 hr. A glass column, 1·5 × 60 cm, was filled with water, and Sephadex was added as a slurry and allowed to settle by gravity; excess fluid escaped through a stopcock at the bottom of the column. The packed column was equilibrated with 0·006 M phosphate buffer, pH 7·4. Ten milliliters of the crude midbrain extract was placed on the column, allowed to penetrate the gel, and rinsed in with 3·0 ml of buffer; elution was with 0·006 M phosphate buffer, pH 7·4. Fifty 5·0-ml fractions were collected using a Gilson Automatic Fraction Collector. The entire procedure may be carried out at room temperature; earlier experiments were conducted at 4°; however, no loss of activity occurred at the higher temperature. This procedure resolved the extract into two components; one inhibited, while the other promoted FFA mobilization in adipose tissue slices. Recently, we have used water to equilibrate and to elute the column; the elution pattern and the activity were unchanged.

Initially, a sample (0·5 ml) of each fraction was tested for lipid mobilization or for inhibition, but with increasing knowledge of the properties of the two components, the number of fractions tested has been reduced. The current procedure is to spot each sample on pH paper; the inhibitor is acid, pH 1·0–2·5, and emerged from the column in fractions 12–20. These results are reproducible, and the elution pattern is consistent from batch to batch of the midbrain extract.

Dowex-1. Further purification of the inhibitor was achieved by chromatography on Dowex-1, used in the acetate form and equilibrated with 0·1 M acetic acid. Ten milliliters of Sephadex inhibitor was placed on the column (65 × 1·5 cm) and developed successively with 0·1, 1 and 3 M acetic acid. Sixty-five 5·0-ml fractions were collected with each solvent. The inhibitor emerged from the column with 0·1 M acetic acid, in fractions 42–57. Thereafter, columns were developed only with 0·1 M acetic acid. Detection of the inhibitor is possible without drying and testing all fractions. The Dowex-1 inhibitor was found to absorb maximally at 248 nm; thus, a sample from each tube was spotted on filter paper, heated for 3 min at 100° and viewed under ultraviolet light. Fractions showing ultraviolet absorption were scanned using a DB-G spectrophotometer; those with maximal absorption at 248 nm were dried under vacuum. The dried inhibitor was white and powdery; the eluate from one column was taken up in 2·0 ml of water. A fine white precipitate was obtained after several hr at 4°; the pH of this fraction was 2·35.

Preparation and incubation of adipose tissue

Male Sprague–Dawley rats, weighing 200–250 g were used in all experiments. Epididymal fat pads were excized under Nembutal anesthesia, rinsed in three changes of saline at room temperature, cut into small fragments and pooled. Samples were chosen at random, each piece was weighed (average weight 60 mg; range 50–70 mg), and placed in a 10·0 ml Erlenmeyer flask containing 1·0 ml of an incubation mixture; unless stated otherwise, the composition was as follows: controls—1·0 ml of 0·06 M phosphate buffer, pH 7·4; norepinephrine—0·10 ml NE (0·2 μg) + 0·90 ml buffer; norepinephrine + inhibitor—0·10 ml NE (0·2 μg) + 0·10 ml inhibitor + 0·80 ml buffer. Phosphate buffer was the carrier for NE and for the inhibitor. Incubation was at 37° in a Dubnoff water bath, with gentle agitation; the gas phase was 95% O_2 and 5% CO_2; no change in pH was observed during the incubation period. After 30 min each tissue was removed, rinsed in saline and blotted dry with gauze; the FFA content was measured. The inhibitor used in these experiments was a Sephadex preparation.

Method of assay

Adipose tissue. The accumulation of FFA within adipose tissue pieces in response to NE was used as the measure of lipid-mobilizing activity; the depression of NE-stimulated FFA release was the criterion for inhibition of lipid mobilization.

Isolated fat cells. In some experiments, the isolated fat cell preparation was used. Fat cells were prepared and incubated according to the method of Rodbell.[7] The FFA content of the medium was determined at the end of a 1-hr incubation period.

Analytical methods

Free fatty acids were measured by the colorimetric method described by Duncombe,[8] with a minor modification by Itaya and Ui.[9] Results for adipose tissue are expressed as μmoles of FFA produced by 100 mg adipose tissue in 30 min, and for isolated fat cells as μmoles of FFA produced by 1·0 ml fat cells in 60 min.

Elemental analysis. C, H, and N-determinations of the Dowex-1 preparation were performed by the Du-Good Chemical Laboratory, St. Louis, Mo.

RESULTS

Effect of inhibitor on NE-stimulated lipolysis in adipose tissue and the isolated fat cell preparation. Lipolytic activity in adipose tissue pieces was increased four-fold in the presence of NE (Table 1); the stimulating action of NE was reduced 62 per cent when the midbrain inhibitor was present in the medium. Lipolysis in the isolated fat cell preparation was increased 12-fold by NE; the action of NE was abolished by the addition of the inhibitor to the incubation system.

Effect of incubation time on inhibitor action. These experiments were conducted to determine at what time during NE-stimulated lipolysis the inhibitor exerted its greatest effect. To this end, the inhibitor was added at varying time intervals after lipolysis with NE had been initiated in (a) adipose tissue, and (b) the isolated fat cell preparation. The inhibitor effect on theophylline-stimulated lipolysis was studied under similar conditions using adipose tissue pieces.

TABLE 1. EFFECT OF INHIBITOR ON NE-INDUCED LIPOLYSIS IN ADIPOSE TISSUE AND IN THE ISOLATED FAT CELL*

Experimental conditions	Adipose tissue (μmoles FFA/100 mg/30 min)	Fat cells (μmoles FFA/ml cells/hr)
Controls	0·31 ± 0·03 (6)†	0·20 ± 0·01 (6)†
NE	1·35 ± 0·09 (6)	2·54 ± 0·02 (9)
NE + inhibitor	0·52 ± 0·06 (6)	0·25 ± 0·02 (9)

* Adipose tissue pieces were incubated in a medium containing 2·5% bovine serum albumin 0·06 M phosphate buffer, pH 7·4; otherwise the assay system was the same as that given under Methods. The FFA content of the tissue was measured after 30 min. Isolated fat cells from 5 g of rat epididymal fat pads were prepared and suspended in Krebs bicarbonate buffer + 2·5% bovine serum albumin, according to the method of Rodbell.[7] The 1-hr incubation was carried out in plastic tubes, as follows: controls—1·0 ml fat cell suspension + 0·20 ml of 0·006 M phosphate buffer, pH 7·4, per tube; NE—1·0 ml fat cell suspension + 0·10 ml NE (0·2 μg) + 0·10 ml phosphate buffer; NE + inhibitor—1·0 ml fat cell suspension + 0·10 ml NE (0·2 μg) + 0·10 ml Sephadex inhibitor. NE and inhibitor were prepared in 0·006 M phosphate buffer, pH 7·4.

† S. E. M.: figures in parentheses represent the number of experiments.

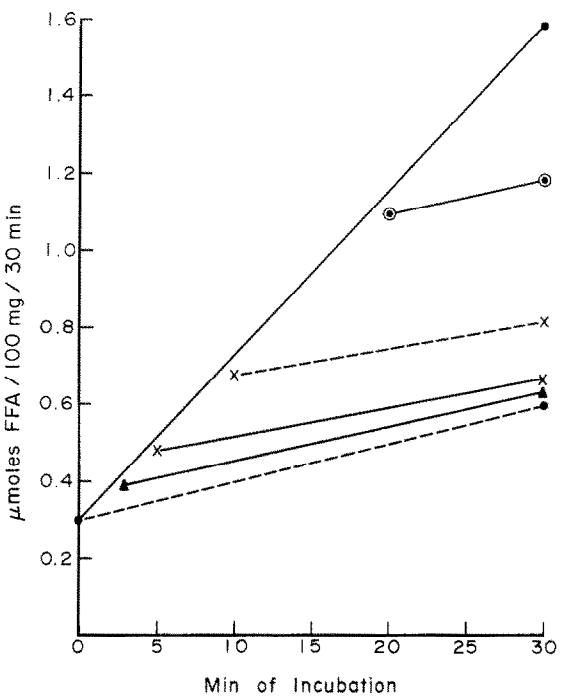

FIG. 1. Adipose tissue—effect of time on inhibitor action toward NE. Adipose tissue fragments were placed in flasks; each contained 0·10 ml NE (0·2 μg) and 0·80 ml of 0·06 M phosphate buffer, pH 7·4; 0·10 ml of inhibitor was added at intervals of 0, 3, 5, 10 and 20 min, with a total incubation time of 30 min. Twelve flasks were prepared for each time interval; six flasks were removed for FFA analysis at the time the inhibitor was added, i.e., 12 flasks were incubated for 3 min, the inhibitor was added to six of these, and incubation was continued; the other six were removed for FFA determination. Six flasks were incubated without inhibitor and contained 0·10 ml NE (0·2 μg) + 0·90 ml buffer; controls (six) contained 1·0 ml buffer. Legend: ●—● no inhibitor; the following represent the intervals at which the inhibitor was added: ●—●, zero min; ▲—▲, 3 min; ×—×, 5 min; ×—×, 10 min; and ⊙—⊙, 20 min.

The inhibitor action on NE-induced lipolysis in adipose tissue is shown in Fig. 1. The inhibitor was added to the incubation system at 0-, 3-, 5-, 10- and 20-min intervals; total incubation time for all samples was 30 min. For example, when the inhibitor was added at zero time, it was present throughout the entire incubation period: addition at 3 min indicates that lipolysis was in progress for 3 min, hence the inhibitor was present for 27 min, and so on.

Inhibition was greatest when the inhibitor was present for the whole incubation period. As NE-induced lipolysis progressed, tissue FFA levels increased and were higher at the time of each inhibitor addition: regardless of the amount of FFA present, or how long lipolysis had been in progress, inhibition always occurred.

Similar results were seen when the inhibitor was added at different time intervals during NE-induced lipolysis, using the isolated fat cell preparation. The pattern of inhibition was identical to that shown in Fig. 1.

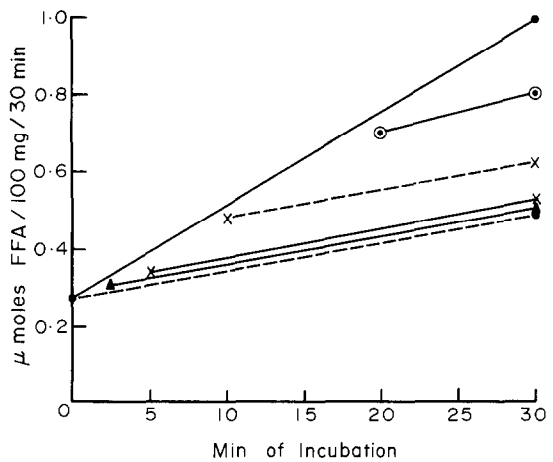

FIG. 2. Adipose tissue—effect of time on inhibitor action toward theophylline. Time intervals and legends are identical to those described in Fig. 1: the incubation system also was similar, except that theophylline, 0·10 ml ($5{\cdot}0 \times 10^{-4}$ M), replaced NE.

The inhibition of theophylline-stimulated lipolysis markedly resembled that seen with NE. The results are shown in Fig. 2. Again, regardless of the time of lipolysis, and the levels of FFA reached, inhibitor addition suppressed further FFA release.

Another series of experiments was carried out to determine if tissue exposed to the inhibitor, then transferred to a medium containing NE, could still carry out FFA mobilization. The results are given in Table 2.

Tissue incubated for 15 min with NE after transfer to inhibitor was capable of a reduced level of FFA mobilization. Tissues exposed to inhibitor were less responsive to the stimulating action of NE; a similar response was elicited in tissues transferred to NE after first being incubated with NE and inhibitor.

Studies in vivo

Effect of inhibitor on NE-induced plasma FFA mobilization in the rat. A six-fold increase in plasma FFA was seen after intraperitoneal injection of NE

TABLE 2. EFFECT OF INCUBATION WITH INHIBITOR ON NE-INDUCED LIPOLYSIS*

Experimental conditions	Medium 1 (μmoles FFA 100 mg)	Medium 2 (μmoles FFA 100 mg)	Increment between 1 and 2	FFA (μmoles 100 mg 30 min)
Experiment 1:				
First medium, NE	0·45 ± 0·02 (6)*			
Second medium, inhibitor		0·61 ± 0·05 (6)	0·16	
Experiment 2:				
First medium, inhibitor	0·31 ± 0·02 (6)			
Second medium, NE		0·47 ± 0·06 (6)	0·16	
Experiment 3:				
First medium, NE + inhibitor	0·34 ± 0·03 (6)			
Second medium, NE		0·49 ± 0·04 (6)	0·15	
Control Experiments:				
Controls				0·32 ± 0·04 (6)
NE				0·87 ± 0·04 (6)
NE + inhibitor				0·41 ± 0·05 (6)

† In Experiment 1, 12 pieces of adipose tissue were incubated in 12 flasks each with the first medium for 15 min; six flasks were removed for FFA determination; the remaining six tissues were rinsed in saline, blotted dry with gauze and placed in the second medium; incubation was for an additional 15 min. Experiments 2 and 3 followed a similar pattern for the two media in each experiment. The incubation systems were: Experiment 1: first medium—0·10 ml NE (0·2 μg) + 0·91 ml buffer; second medium—0·10 ml inhibitor + 0·90 ml buffer; Experiment 2: first medium—0·10 ml inhibitor + 0·90 ml buffer; second medium—0·10 ml NE + 0·90 ml buffer; Experiment 3: first medium—0·10 ml NE + 0·10 ml inhibitor + 0·80 ml buffer; second medium—0·10 ml NE + 0·90 ml buffer. In control experiments, viz. controls, NE and NE + inhibitor, adipose tissue pieces were incubated for 30 min as described under Methods.

* S. E. M.; figures in parentheses represent the number of experiments.

TABLE 3. EFFECT OF INHIBITOR ON RAT PLASMA FREE FATTY ACIDS*

Experimental conditions	Plasma (μmoles FFA/ml)	Less controls	Tissue (μmoles FFA/100 mg/30 min)	Less controls
Controls	0·13 ± 0·05 (5)†		0·84 ± 0·05 (5)	
NE	0·77 ± 0·06 (5)	0·64	2·35 ± 0·45 (5)	1·52
NE + inhibitor	0·52 ± 0·06 (5)	0·39	1·14 ± 0·21 (5)	0·30

* Under Nembutal anesthesia, each animal was injected, intraperitoneally, as follows: controls 0·60 ml of 0·006 M phosphate buffer, pH 7·4; NE 0·20 mg NE in 0·60 ml phosphate buffer; NE + inhibitor 0·10 ml NE (containing 0·2 mg) + 0·50 ml inhibitor, pH 7·4. Blood was removed by cardiac puncture, 5 min post-injection, and placed in tubes containing heparin as anticoagulant. Blood samples were stored in an ice bath until they were centrifuged in a refrigerated centrifuge. Plasma (1·0 ml) was used for FFA determination. Epididymal fat pads were excised immediately after bleeding, and placed in saline; the thin distal portions of fat pads from each group were pooled; five pieces from each group were selected randomly and incubated in 1·0 ml of 0·06 M phosphate buffer, pH 7·4, for 30 min. The FFA content of the tissues was measured.

† S. E. M.; figures in parentheses represent the number of animals.

(0·2 mg) into the anesthetized rat. Administration of NE and inhibitor, simultaneously, resulted in a 30 per cent decrease in plasma FFA, compared to NE alone (Table 3). Pieces of epididymal fat pads from the same animals were incubated for 30 min in phosphate buffer. Tissues from NE-treated animals accumulated three times as much FFA as control tissues, while adipose tissues from animals that had received NE plus inhibitor contained 80 per cent less FFA than the NE-treated group at the end of the incubation period.

Effect of inhibitor on NE-stimulated FFA mobilization and on blood pressure in the dog. Plasma FFA levels increased under the influence of NE, from 266 to 1135 μmoles FFA/l. within 10 min after infusion with NE (Table 4); the high level was maintained throughout the 30-min period. Infusion with NE and inhibitor followed, and plasma FFA levels fell to that of the controls; this fall occurred despite the simultaneous infusion of NE. After return to NE infusion, a marked elevation of plasma FFA occurred; an increase of 2·5-fold was evident at the first 10-min sampling.

A moderate increase in blood pressure was observed during infusion with NE, from a control level of 130–160 mm Hg. Infusion with NE and inhibitor was begun after blood pressure had returned to control levels. During simultaneous infusion of NE and inhibitor, blood pressure varied little, and was maintained at 130 mm Hg throughout the infusion period. The return to NE infusion resulted in an increase in blood pressure to 150 mm Hg, only slightly below that seen with the initial NE administration.

Some properties of the Dowex-1 inhibitor

In Table 5, we have used the Sephadex fraction as our starting point; the crude midbrain extract contains both an activator and an inhibitor of FFA mobilization. Both fractions of inhibitor were prepared from the same midbrain extract; at the concentrations tested, the Sephadex fraction and the Dowex-1 fraction inhibited FFA mobilization in adipose tissue by 94 and 66 per cent respectively. The degree of purification of the Dowex-1 fraction is not known, since we are uncertain of the nature of the inhibitor; however, the extent of purification may be assessed from Table 6, where we have given the protein nitrogen content of the three fractions of

TABLE 4. EFFECT OF INHIBITOR ON NE-INDUCED FREE FATTY ACID MOBILIZATION AND ON BLOOD PRESSURE IN THE DOG*

Experimental conditions	Plasma (μmoles FFA l)	Blood pressure (mm Hg)
Pre-saline infusion	240 ± 24†	130/90
Post-saline infusion	266 ± 34	130/90
Norepinephrine infusion:		
10 min	1135 ± 221	
20 min	995 ± 171	160/104
30 min	1442 ± 247	
Inhibitor + norepinephrine infusion:		
10 min	299 ± 45	
20 min	265 ± 53	130/95
30 min	345 ± 61	
Norepinephrine infusion:		
10 min	845 ± 121	
20 min	1140 ± 104	150/100
30 min	1065 ± 151	

* Four mongrel male dogs were anesthetized with Nembutal. Blood pressure and ECG were monitored throughout the experiments by an E & M Physiograph, model 4A. Infusions were given via the right femoral vein; blood samples were removed from a cannula in the left femoral artery. Normal saline was infused for 10 min; blood samples were taken before and after saline infusion. NE in saline was given as an infusion of 1·0 μg/kg/min for 30 min; blood was sampled at 10-min intervals. Infusion with inhibitor containing NE (1 μg/kg/min) was started after blood pressure had returned to normal, and continued for 30 min, with blood sampling at 10-min intervals. The NE + inhibitor was discontinued, and NE again was infused; blood was removed at 10-min intervals for 30 min. Blood was placed in tubes containing heparin as the anticoagulant; the tubes were stored in ice, and centrifuged at 10,000 rev/min in a refrigerated centrifuge. Plasma (1·0 ml) was used for FFA determination.

† S. E. M.

the midbrain extract. The low protein content of the Sephadex fraction (1·6% of that of the crude extract) indicated that this step alone resulted in a major purification. The absence of protein from the Dowex-1 preparation suggested a high degree of purity, a view which was strengthened by the ease of crystallization of this fraction.

Elemental analysis of a sample of Dowex-1 inhibitor gave the following composition: C, 31·64%; H, 4·42%; N, 20·66%; and ash, 20·56%. The high ash content made oxygen calculation by difference impossible; it suggested that the inhibitor may possess a heavy component such as a metal.

TABLE 5. INHIBITORY ACTION DURING PURIFICATION PROCEDURE*

Experimental conditions	(μmoles FFA/100 mg/30 min)	Less controls	Per cent inhibition
Controls	0·37		
Norepinephrine	0·72	0·35	
NE + Sephadex inhibitor	0·35	0·02	94
NE + Dowex-1 inhibitor	0·49	0·12	66

* Assay system for controls, NE and NE + Sephadex inhibitor is identical to that given under Methods; the Dowex-1 incubation system contained 0·10 ml NE (0·2 μg) + 0·10 ml Dowex-1 inhibitor + 0·80 ml phosphate buffer. The above figures represent average values for three experiments.

TABLE 6. PROTEIN NITROGEN CONTENT OF INHIBITOR FRACTIONS*

Fraction	Protein nitrogen (mg ml)
Crude midbrain extract	7·34
Sephadex inhibitor	0·115
Dowex-1 inhibitor	0

* Protein nitrogen was determined by the method of Lowry *et al.*[19]

DISCUSSION

Inhibition of FFA mobilization may occur (a) by inactivation of a lipolytic enzyme in adipose tissue, or (b) by re-esterification of the FFA produced during lipolysis; either would result in diminished FFA release.

The inhibitor described in this publication depressed NE-stimulated FFA mobilization in adipose tissue slices, and the isolated fat cell; the rise in plasma FFA levels associated with NE administration also was suppressed.

That the decreased FFA release in adipose tissue was not an artifact was verified in the intact animal. In the rat, simultaneous injection of NE and inhibitor suppressed FFA mobilization, as reflected in the lowered plasma FFA levels, and the reduced ability of adipose tissue from NE plus inhibitor-treated animals to mobilize FFA in the incubation system.

A fact brought out by the dog experiments was the reversibility of the inhibitor action. Infusion of NE and inhibitor abolished the FFA-mobilizing action of NE, as evidenced by the fall in plasma FFA levels. However, a return to NE infusion completely reversed the inhibitor action; plasma FFA were elevated to the level seen with the initial administration.

The reversibility of the inhibitor action was not demonstrable *in vitro*. FFA release in adipose tissue exposed to inhibitor or to inhibitor plus NE was not stimulated significantly when placed in a medium containing NE. Isolated pieces of adipose tissue may not possess a means to inactivate, or to rid itself of the inhibitor.

A coincidental finding was the action on blood pressure. The rise in blood pressure seen with NE infusion was abolished when NE and inhibitor were administered together. Like the reversal of FFA inhibition, the effect on blood pressure also was reversed upon return to NE infusion. This suggested either inactivation of the inhibitor, or that the inhibitor had occupied receptor sites from which it could be displaced easily by an excess of NE. An alternative explanation need not involve actual displacement of inhibitor from receptor sites, but simply the occupation of more sites by NE, thereby vitiating the action of the inhibitor.

A parallelism existed between the time the inhibitor was present in the incubation medium and the extent of inhibition. In studies where lipolytic activity had proceeded from 3 to 20 min before inhibitor addition, it was evident that the inhibitor could affect FFA release at any time. The pattern of inhibition was the same whether the FFA-mobilizing agent was NE or theophylline. FFA release did not cease entirely when the inhibitor was added, nor did it reach the level attained by tissues incubated without inhibitor.

The inhibitor may exert its action by competing with NE for receptor sites. Thus, if the lipolytic action of NE is mediated through the activation of the membrane enzyme adenyl cyclase,[11] a possible site of action of the midbrain inhibitor may be at the level of adenyl cyclase. The action could be either inhibition of the enzyme, or merely occupation of the NE receptor site.

Theophylline inhibits the enzyme phosphodiesterase which degrades cyclic 3′,5′-AMP.[12] The stimulation of lipolysis by theophylline is believed to be due to the accumulation of cyclic AMP. The suppression of theophylline-stimulated lipolysis by the midbrain inhibitor could be due either to inhibition of the already formed cyclic AMP, or to its action on adenyl cyclase. Steinberg[13] and Stock *et al.*[14] have suggested that inhibition of theophylline-stimulated lipolysis by prostaglandin E_1 may be due to inhibition of the enzyme adenyl cyclase, rather than to an inhibitory action on the cyclic AMP already present.

Experiments are in progress to elucidate the mechanism of action of the inhibitor, and to determine its effect on blood lipids, particularly triglycerides and cholesterol. Methods have been developed for further purification and characterization of the midbrain inhibitor.

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